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Combinatorial coding of *Drosophila* muscle shape by Collier and Nautilus

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ABSTRACT

The diversity of *Drosophila* muscles correlates with the expression of combinations of identity transcription factors (iTFs) in muscle progenitors. Here, we address the question of when and how a combinatorial code is translated into muscle specific properties, by studying the roles of the Collier and Nautilus iTFs that are expressed in partly overlapping subsets of muscle progenitors. We show that the three dorso-lateral (DL) progenitors which express Nautilus and Collier are specified in a fixed temporal sequence and that each expresses additionally other, distinct iTFs. Removal of Collier leads to changes in expression of some of these iTFs and mis-orientation of several DL muscles, including the dorsal acute DA3 muscle which adopts a DA2 morphology. Detailed analysis of this transformation revealed the existence of two steps in the attachment of elongating muscles to specific tendon cells: transient attachment to alternate tendon cells, followed by a resolution step selecting the final sites. The multiple cases of triangular-shaped muscles observed in *col* mutant embryos indicate that transient binding of elongating muscle to exploratory sites could be a general feature of the developing musculature. In *nau* mutants, the DA3 muscle randomly adopts the attachment sites of the DA3 or DO5 muscles that derive from the same progenitor, resulting in a DA3, DO5-like or bifid DA3-DO5 orientation. In addition, *nau* mutant embryos display thinner muscle fibres. Together, our data show that the sequence of expression and combinatorial activities of Col and Nau control the pattern and morphology of DL muscles.

Introduction

"It takes 47 different muscles to frown and only 13 to smile". This popular saying illustrates the diversity of muscles needed for simple coordinated movements. The genetic and molecular mechanisms that build up this diversity remain, however, poorly understood. The *Drosophila* larval musculature, made of a stereotyped array of around 30 different muscles in each hemi-segment, is an ideal model to study this process (Bate, 1993). Each individual muscle is composed of a single multinucleated syncytial fibre, characterised by its position and orientation with respect to the dorso-ventral (D/V) and antero-posterior (A/P) axes, size and number of nuclei, epidermal attachment sites and ultimately, innervation. These unique properties are collectively referred to as muscle identity. The *Drosophila* muscle pattern is seeded by a special class of myoblasts, called founder cells (FCs), which display the unique property of being able to undergo multiple rounds of fusion with another class of myoblasts, the Fusion Competent Myoblasts (FCMs). FCs originate from the asymmetric division of progenitors cells (PCs), selected from equivalence groups of myoblasts, called promuscular clusters, via Notch (N)-mediated lateral inhibition and short range receptor tyrosine kinase (RTK) signalling (Buff et al., 1998; Carmena et al., 1995). Promuscular clusters are themselves specified at fixed positions within the somatic mesoderm, in response to positional information issued from the ectoderm and provided by long range Wingless and Dpp signalling (Carmena et al., 1998).

Muscle diversity is first revealed by the unique patterns of "identity" transcription factors (iTFs) that accompany progenitor segregation (reviewed in Frasch, 1999; Tixier et al., 2010). The expression of a particular iTF persists in only one of the two sibling FCs derived from the division of a progenitor, such that the properties unique to each muscle could reflect the specific combination of iTFs expressed in its PC and maintained in its FC (Bate and Rushton, 1993; Baylies et al., 1998; Bourgouin et al., 1992). However, the concept of combinatorial control of muscle identity relies, so far, upon the compared expression and function of individual iTFs. Here, we address the question of when, and how the cumulative and/or combinatorial activity of several iTFs does act during the muscle specification process, using, as a paradigm, the Dorsal/Acute 3 (DA3) muscle, which originates from a PC expressing Nau and Col (Crozatier and Vincent, 1999; Dubois et al., 2007; Keller et al., 1998). Nau is the single *Drosophila* ortholog of the mammalian family of bHLH myogenic regulatory factors (MRFs) that are at the core of the myogenic regulatory network (Michelson et al., 1990; Paterson et al., 1991; Sambasivan

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and Tajbakhsh, 2007 and Weintraub et al., 1989 for review of MRFs). Nau function has been the subject of conflicting reports. On one side, it was proposed that Nau exerts general myogenic functions, similar to vertebrate MRFs (Misquitta and Paterson, 1999; Wei et al., 2007). On the other side, muscle-specific defects observed in *nau* mutant embryos suggested that Nau acts as an iTF (Balagopalan et al., 2001; Keller et al., 1998). Col is the single *Drosophila* member of the COE (Collier/Early B-Cell Factor) family of transcription factors (Dubois and Vincent, 2001; Daburon et al., 2008). *Ci-coe* has recently been shown to be a critical determinant of atrial siphon muscle fate in the ascidian *Ciona intestinalis* (Stolfi et al., 2010) but the functions of *ebf/coe* genes in vertebrate myogenesis remain little known. EBF(s) could contribute to the transcriptional regulation of *Xenopus* muscle development, in part via a positive feedback loop between EBF and MyoD (Green and Vetter, 2011). *Drosophila* Col is expressed in a large promuscular cluster at the origin of several PCs and this expression is maintained in a single muscle, the DA3 muscle, where it is required for normal development (Crozatier and Vincent, 1999). We have previously shown that promuscular *col* activation is controlled by an "early" cis-regulatory module (CRM) that integrates positional information and Notch-mediated lateral inhibition during the process of PC selection. A separate, "late" CRM then takes over and maintains robust *col* transcription in the DA3/DO5 PC and DA3 muscle lineages. This relay both depends upon Hox information (Enriquez et al., 2010) and direct binding of Col, revealing a handover mechanism at the PC stage (Dubois et al., 2007; Enriquez and Vincent, 2010). Finally, examination of *col* transcription in *nau* mutants showed that Nau is essential for robust *col* activation in the nuclei of FCMs that fuse with the DA3 FC (Dubois et al., 2007). We further explore here the respective roles of Col and Nau in conferring the DA3 muscle specific properties.

We first show that Col and Nau are expressed together in the three PCs at the origin of all the dorso-lateral (DL) muscles, the DA3, DO3, DO4, DO5, DT1 and LL1 muscles (Beckett and Baylies, 2007; Nose et al., 1998) and that these progenitors are born sequentially. Each expresses a specific combination of iTFs that results, in part from cross-regulation already occurring at this stage. In *col* mutant embryos, all the DL muscles show specific changes in their epidermal insertion sites, showing that, even when transient, Col expression is required for DL PC identity. Detailed analysis of the DA3>DA2 muscle transformation that is observed in absence of Col revealed that the final orientation of the DA3 muscle involves two steps: a transient attachment to several epidermal sites, followed by a resolution step selecting the definitive sites. The multiple other cases of triangular-shaped DL muscles observed in *col* mutant embryos indicate that transient binding of elongating muscle to exploratory sites could be a general feature of the developing musculature. In *nau* mutant embryos the DA3 shows transformation towards its sibling, DO5 muscle, with many cases of bifid DA3/DO5 fibres, a phenotype aggravated in *nau/col* hypomorphic conditions. In addition we find that all myofibres are thinner in *nau* than wt embryos, showing that *nau* activity controls both generic and muscle-specific differentiation programmes. The early Col function in specifying DL progenitor identity, general function of Nau in ensuring proper fibre size and combined activities of Nau and Col in the DA3 muscle differentiation process provide a clear example of combinatorial transcriptional coding of muscle-specific shapes.

Materials and methods

Drosophila genetics

The following Drosophila mutant alleles and transgenic constructs were used: col^1 (Crozatier et al., 1999), *Pcol85-Gal4, UASmCD8GFP* (*col>GFP* (Krzemien et al., 2007)), *P9Gal4, UASmCD8GFP (P9cG>GFP* (Dubois et al., 2007)), CRM276-LacZ and 4_0.9-LacZ (Enriquez et al., 2010), nau^{CK188} (Balagopalan et al., 2001), *UAS-col* (Vervoort et al., 1999), *twist-Gal4* (Baylies and Bate, 1996), $Poxm^{R361}$ (Duan et al., 2007), $Kr^{CD+}Kr^1$ (Romani et al., 1996), $slou^{286}$ (Knirr et al., 1999). Mutant alleles and transgenic constructs were balanced over marked chromosomes: *Cyo twist-lacZ; TM3 twist-lacZ; Cyo dfd-EYFP; TM6b dfd EYFP*. All Gal4-UAS crosses were performed at 25 °C. The strain w^{118} was used as wt reference.

Plasmid constructions and transgenic lines

A HA tag was inserted in frame at the N-terminus of the Col open reading frame, before cloning the full length *col* cDNA into the PUAS vector ($PUASCol^{HA}$). The upstream 9_0.9 *col* genomic fragment (Dubois et al., 2007) was inserted into the *attB-inslacZ* vector (Enriquez et al., 2010). The Zh8 AttP platform at position 49D on the second chromosome was used for site-specific insertion (Bischof et al., 2007).

Immunohistochemical staining and imaging

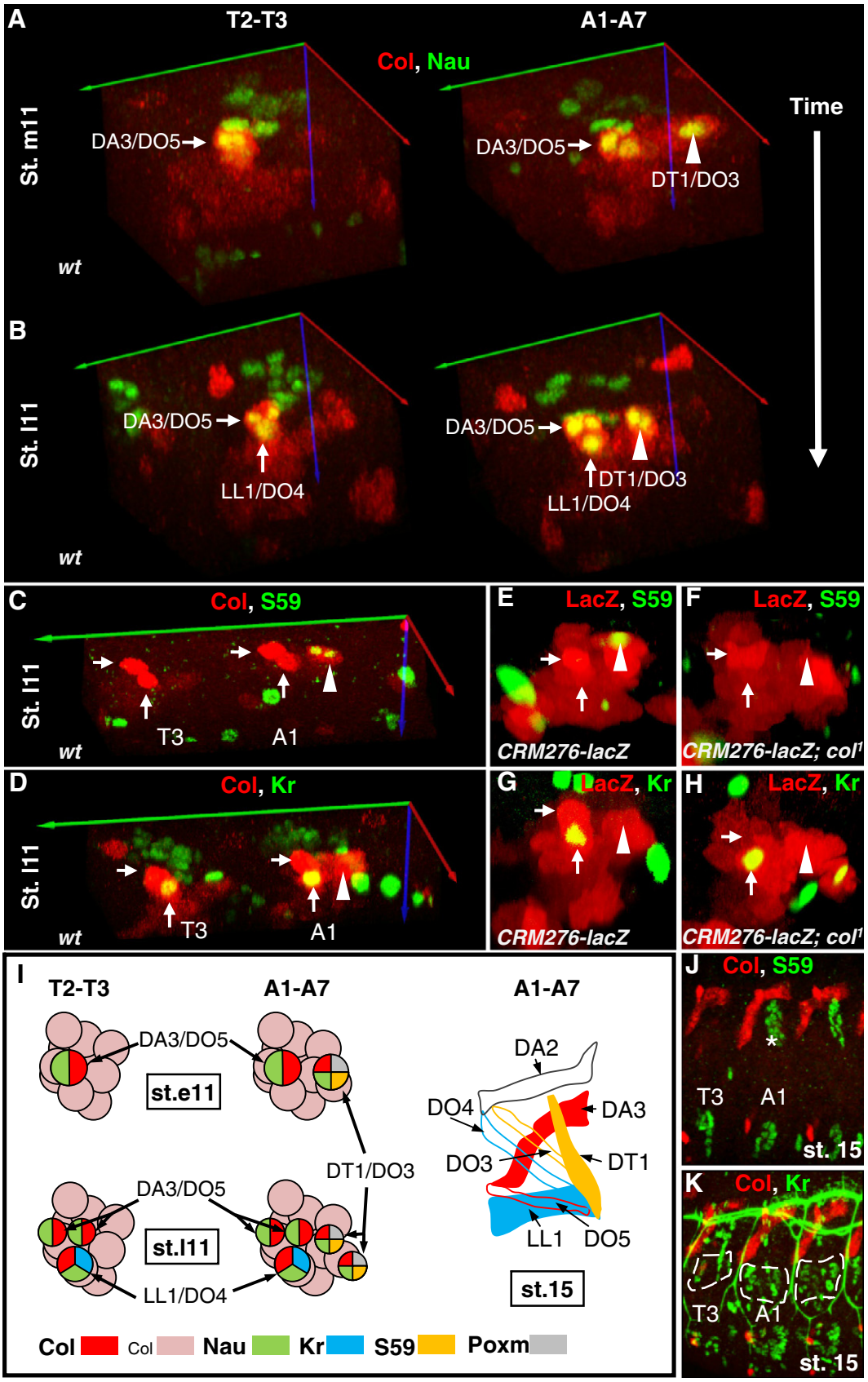
Embryos were fixed and processed for antibody staining as described (Crozatier et al., 1996). Primary antibodies were: mouse anti-Col (1/100) (Dubois et al., 2007), anti-β-galactosidase (Promega, 1/1000) and anti α-PS2 integrin, (Developmental studies Hybridoma Bank, 1/5); rabbit anti-Mef2 and anti-Nau (1/100) (provided by E. Furlong, Heidelberg, Germany and B. Paterson, Bethesda, MD, USA, respectively), anti-Kr (Gaul et al., 1987), anti S59 (Dohrmann et al., 1990); anti-Vg (provided by AJ. Simmonds, Edmonton, Canada); Secondary antibodies were: Alexa Fluor 488-conjugated goat anti-rabbit and goat anti-mouse; Alexa Fluor 555-conjugated goat anti-rabbit and goat anti-mouse; Alexa Fluor 647-conjugated goat anti-mouse (all Molecular Probes, 1/300); and biotinylated goat anti-mouse (Vector Laboratories, 1/1000). For staining with Phalloidin, embryos were manually devitellinized to avoid methanol treatment, which destabilises the cytoskeleton. 3-D reconstructions of the topology of DL progenitor and founder cells (see Fig. 1 A–H and S1,2) and muscles (see Fig. S3) were made from 200 to 500 nm thin sections acquired on a Leica SP5 confocal microscope at ×40 magnification, numerical zoom 4×, using Amira, (Visage Imaging GmbH) and Volocity (PerkinElmer) software.

SEM analysis of embryonic muscle pattern

For SEM analysis, *col* and *nau* mutant strains were balanced over the marked balancers Cyo Dfd-EYFP, and Tm6B Dfd-EYFP, respectively. Embryos were allowed to develop at 25 °C. After removal of the chorion by bleaching, live mutant embryos were selected under a Leica stereomicroscope equipped for epifluorescence. Stage-15 embryos were hand

Fig. 1. Expression of Col, Kr, Nau and S59 defines a dorso-lateral muscle progenitor code. (A,B) Col and Nau expression (yellow) between mid-stage 11 (A) and late stage 11 (B), in T (left) and A segments (right). (A) The newly born DA3/DO5 FCs and DT1/DO3 PC/FCs are indicated by a horizontal arrow and vertical arrowhead, respectively. (B) The DT1/DO3 PC has divided, and an additional progenitor (vertical white arrow) selected in both T and A segments. (C,D) Expression of Col (red) and either S59 (C) or Kr (D) (green) in late stage 11 embryos, T3 and A1 segments. The DT1 and DO3 PC/FCs express S59 while the ventral-most progenitor expresses Kr. (E,F) S59 and (G,H), Kr expression (green) in mid (E,F) and late (G,H) stage 11 wt (E,G) and col^1 (F,H) embryos carrying the *CRM276-LacZ* transgene. LacZ staining visualises the Col expressing cluster and progenitors (I) Schematic representations. Left: temporal sequence of specification of the DA3/DO5, DT1/DO3 and LL1/DO4 PCs in T and A segments. Low and high levels of Col are indicated in pink and red, respectively, Nau in green, Kr in blue, S59 in yellow and Poxm in grey. Right: The corresponding muscle fibres at stage 15, indicating maintenance of Col, Kr and S59 expression in the DA3, LL1 and DT1 muscles, respectively (see Dohrmann et al., 1990; Ruiz-Gomez et al., 1997). (J, K) Immunostaining of stage 15 embryos for Col (red) and S59 or Kr (green). Positions of the T3 and A1 segments are indicated. (J) The DT1 muscle, indicated by a white asterisk, is specific to A segments. (K) The LL1 muscle is circled by a dotted line. In A–D, the green, red and blue arrows indicate the antero-posterior (A/P), dorso-ventral (D/V), and medio-lateral (ML) axes, respectively.

devitellinized, and fixed on their dorsal side on a coverslip coated with polylysine. They were then dissected and the fillets stretched to expose the somatic muscles. Fillets were fixed in a double aldehyde mixture (4% formaldehyde, 2.5% glutaraldehyde in 1× PBS), washed in water, and dehydrated through ethanol series. Following HMDS (hexamethyldisilazane) drying, fillets were sputtered with a gold-palladium coat



(JFC 1100 Jéol), and examined with either a Hitachi S450 microscope or TM-1000 tabletop microscope.

Results

Sequential specification of the three dorso-lateral muscle progenitors

Col is first expressed in the somatic mesoderm at embryonic stage 10, in a large cluster of myoblasts. We reported previously that this cluster gives rise to the DA3/DO5 and DT1/DO3 PCs in the T2-A7 segments and A1-A7 segments, respectively (Crozatier and Vincent, 1999; Enriquez et al., 2010). Based on reporter gene expression, we initially proposed that the second PC gave rise to the DT1 and DO4 muscles (Crozatier and Vincent, 1999), but we now favour the DT1/DO3 lineage, as proposed by (Carmena et al., 1995). The Col-expressing PCs also express Nau, a general marker of most if not all PCs and FCs (Keller et al., 1998; Michelson et al., 1990; Wei et al., 2007). The DA3/DO5 and DT1/DO3 PCs divide asynchronously, the DA3/DO5 PC dividing at mid-stage 11 (Fig. 1A) and the DT1/DO3 at late-stage 11 (Fig. 1B). When examining in detail this sequence of divisions, we noticed in late stage 11 embryos the appearance of another, large size cell, expressing both Col and Nau in the T2-A7 segments (Fig. 1B). The position of this cell, immediately ventral and internal to the DA3 and DO5 FCs, suggested that it could be an additional PC selected from the Col-expressing cluster, slightly ventral and later than the DA3/DO5 PC. Consistent with a PC identity, this cell divides into two smaller cells at the beginning of stage 12 (Fig. S1). To determine its molecular identity, we double-stained embryos for Col and either S59 or Krüppel (Kr), expressed in the DT1/DO3 and LL1/DO4 progenitors, respectively (Carmena et al., 1995; Dohrmann et al., 1990; Ruiz-Gomez et al., 1997). These double stainings confirmed the co-expression of S59 and Col in the DT1/DO3 PC (Fig. 1C) and showed that the PC that is selected late from the Col-expressing cluster expresses also Kr (Fig. 1D). It therefore corresponds to the LL1/DO4 muscle progenitor (Ruiz-Gomez et al., 1997).

Precisely timed immuno-staining experiments thus revealed that Col is expressed in all three PCs at the origin of the DL muscles (DA3, DO5, DT1, DO4, LL1, DO3, (Nose et al., 1998); Fig. 2A) and revealed that these PCs are specified in a fixed temporal sequence. At their birth time, each DL PC already expresses a specific code of iTFs in addition to Nau, either Col (DA3/DO5), Col/Kr (LL1/DO4) or Col/S59/Poxmeso (Poxm) (DT1/DO3), (Fig. 1I (Carmena et al., 1995; Cox and Baylies, 2005; Dohrmann et al., 1990; Duan et al., 2007)), suggesting that both positional and temporal clues could be involved in specification of their identity.

Epistatic relations between Col and other iTFs in dorso-lateral progenitors

The iTF code that is specific to each DL PC could result from epistatic or cross-repressive interactions between different iTFs, as observed in some other lineages (Jagla et al., 2002; Knirr et al., 1999; Lord et al., 1995). We therefore looked at S59 and Kr expression in *col*^{1} null mutant embryos. LacZ expression under the control of the early *col* CRM (CRM276) served to visualise the Col-expressing cluster and progenitors (Enriquez et al., 2010). S59 expression is lost from the DT1/DO3 PC, and therefore is downstream of Col in this lineage (Fig. 1E,F). S59 expression in the DT1 muscle precursor has recently been shown to be also dependent upon Poxm (Duan et al., 2007). We found that Poxm expression is specifically lost from the DT1/

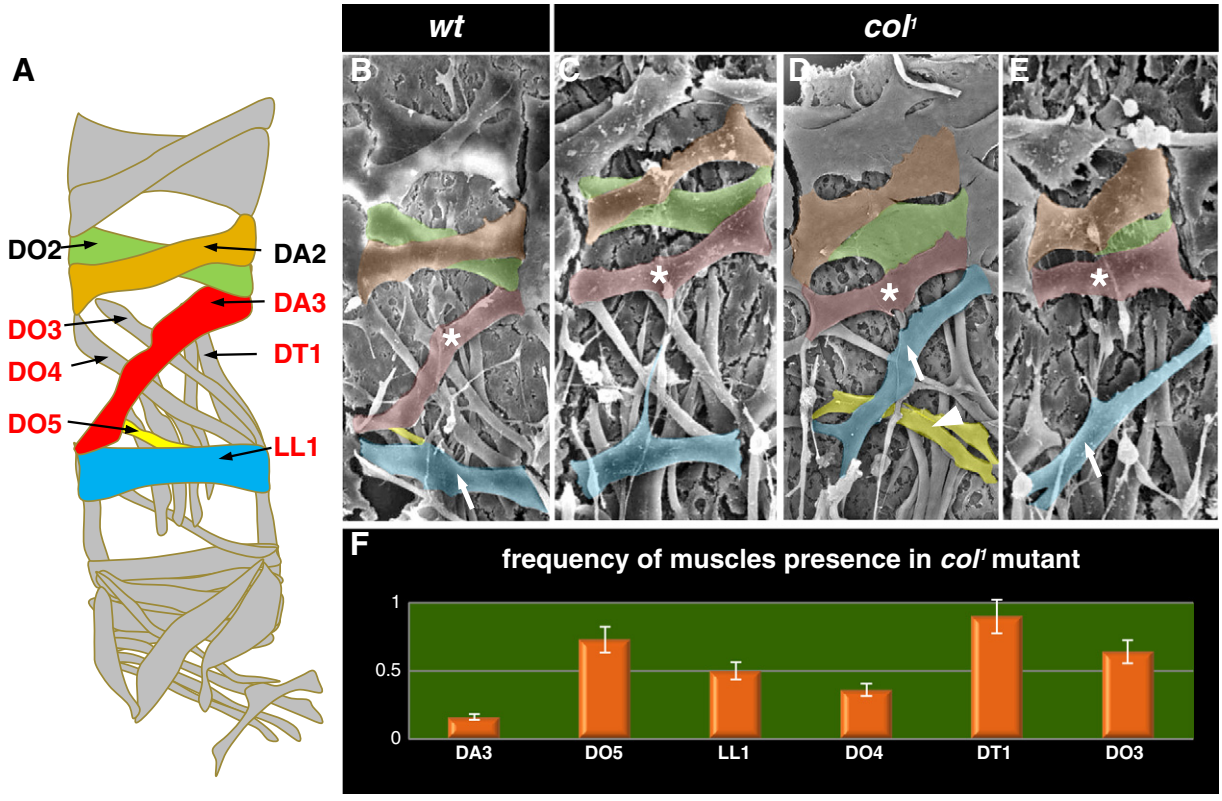


Fig. 2. Muscle phenotypes of *col* mutant embryos. (A) Schematic diagram of the muscle pattern in A2–A7 segments adapted from (Bate, 1993). (B–E) EM-scanning views of the abdominal musculature in stage 16 embryos. The internal face of one wt (B) and *col*^{1} (C–E) segment is shown. The D02, DA2, DA3, DO5 and LL1 muscles are colour-coded as in A. (C) The DA3 muscle (white asterisk) is transformed into a DA2-like muscle. (D) The LL1 muscle (white arrow) is transformed into a DA3-like muscle. In addition, the DO4 muscle (white arrowhead) adopts a DO5 morphology, while the DO3 muscle is also abnormal. Since SEM analysis cannot distinguish between the DO5 and transformed DO4 muscles, both are coloured in yellow. (E) The DA3 and LL1 muscle are transformed into DA2-like and DA3-like muscles, respectively, while the DO3, DO4 and DO5 muscles are absent. (F) Histogram representing the fraction of *col1* segments in which each indicated muscle forms normally.

DO3 progenitor in *col* mutant embryos and may therefore be an intermediate of S59 regulation by Col (Fig. S2). By contrast, Kr expression in the LL1/DO4 PC is unaffected in *col* mutant embryos and is therefore independent of Col activity (Fig. 1G,H). Inversely, Col expression is normal in either, *Kr, Pxm* or *S59* mutants (not shown). Thus Col acts upstream of Poxm and S59 in defining the DT1/DO3 progenitor identity. It regulates its own expression in the DA3/DO5 PC and, subsequently, the DA3 myofibre (Crozatier and Vincent, 1999; Enriquez et al., 2010). The restriction of Col auto-regulation to the DA3/DO5 PC suggests that this handover mechanism is dependent upon (an) other, still unknown, either activating or repressing TFs that distinguish the DA3/DO5 PC from the other DL PCs. Together, these expression data show that the molecular identity of DL muscles reflects sequences of regulations between different iTFs at the PC stage, which are specific to each PC.

Morphological transformations of the dorso-lateral muscles in col mutant embryos

While Kr and S59 remain expressed in the LL1 and DT1 muscles, respectively, Col expression is only maintained in the DA3 myofibre (Fig. 1J,K; (Dohrmann et al., 1990; Ruiz-Gomez et al., 1997)). Our analysis of the *col* mutant phenotype therefore initially focused on the DA3 muscle (Crozatier and Vincent, 1999). In order to determine which other muscles were affected, and in the absence of specific markers for the DO3, DO4 and DO5 muscles (Tixier et al., 2010), we performed morphological analyses of stage 15 embryos, using Scanning Electron Microscopy (SEM) (Fig. 2B–E) and phalloidin staining of the acto-myosin cytoskeleton of stage 17 embryos (Fig. S3). This first level of analysis revealed that all the DL muscles (Fig. 2A) were either missing or mis-specified in *col* mutant embryos at a statistically significant frequency (Fig. 2F), while the more dorsal or more ventral muscles were not affected.

The DA3 is the most often affected muscle. In 84% of *col* mutant segments (N = 48/57), it is replaced by a muscle at a more dorsal position and oriented parallel to the DA2 muscle, suggestive of a DA3 > DA2 transformation (Fig. 2C–E). The DA3 sibling, the DO5 muscle is present in 73% (N = 42/57) of segments. The LL1 and DO4 muscles are present in 51% (N = 29/57) and 36% (N = 21/57) of col^1 embryonic segments, respectively (Fig. 2F). We observed segments displaying both a DA3 > DA2 transformation and a DO5 muscles while a muscle at the position and with the same orientation than the DA3 muscle was present, suggesting a complex set of muscle transformations. Every time that both a DA3 > DA2 and a DA3-like muscles are present, the LL1 muscle is missing (16% of mutant embryos, N = 9/57; Fig. 2D, E). We conclude that the DA3-like muscle corresponds to a LL1 > DA3 transformation. In support of this interpretation, we observed muscles of intermediate morphology, i.e., displaying both the normal LL1 epidermal attachment sites and a supplementary projection towards the DA3 attachment site, a phenotype that we interpret as a partial LL1 > DA3 transformation (8%, N = 5/57; Fig. 2C and S3). Finally, two muscles oriented like the DO5 are present in some col^1 segments when the DO4 muscle is missing (Fig. 2D), indicating a re-orientation of the DO4 muscle parallel to DO5 (21% of mutant segments, N = 12/57). Similar to partial LL1 > DA3 transformations, we observed cases of DO4 muscles projecting additional, thin extensions towards the anterior attachment site of the DO5 muscle, suggestive of partial DO4 > D05 transformations (7%, N = 4/57; Fig. S3B,C). The DO3 and DT1 muscles originate from a PC that is only specified in abdominal segments (Crozatier and Vincent, 1999; Dohrmann et al., 1990; Enriquez et al., 2010). A DT1 muscle forms normally in about 90% of col^1 mutant segments (N = 46/51), while a DO3 muscle forms in 64% of segments (N = 33/51; Fig. 2F).

In summary, morphological observations show that the entire pattern of DL muscles is disrupted in *col* mutant embryos. At this level of analysis, the most obvious defect is mis-orientation of many myofibres, suggesting that in absence of Col activity, insertion site choice is often changed. While all DL muscles are affected to some extent, the most frequently observed defects are DA3 > DA2, LL1 > DA3, and DO4 > DO5 transformations, which indicate changes in progenitor identity.

Changes in muscle attachment sites upon loss or gain of Col activity

To further characterise the muscle transformations resulting from the loss of Col activity, we used specific muscle markers such as Vestigial (Vg) and Kr, which are expressed in the DA1, DA2, DA3, and LL1, and the DA1, LL1 and LT4 muscles, respectively (Fig. 3A,D; (Bate et al., 1993; Ruiz-Gomez et al., 1997)). The *col 4_0.9 lacZ* reporter gene served as a DA3 identity marker (Enriquez et al., 2010). Double Vg/LacZ staining of col^1 mutant embryos confirmed that the DA3 muscle adopts a DA2-like morphology (97% of segments at stage 15; N = 96/99), while the DA2 muscle is itself unaffected. A DA3 > DA2 transformation is already observed in hypomorphic *col* mutants ($Pcol > GFP/col^1$) where *GFP* recapitulates the *col* expression pattern (Fig. 3C, Krzemien et al., 2007). Vg staining of col^1 embryos also confirmed that the LL1 muscle adopts a DA3 morphology in at least 10% of segments (N = 8/77; Fig. 3B). The loss of Vg expression at the expected position for the LL1 or LL1 > DA3 in more than half of the segments (Fig. 3B; N = 43/77) could hide, however, other phenotypes. We therefore turned to Kr antibody staining, since Kr expression in the LL1 muscle does not depend upon Col activity. The Kr expression pattern confirmed that the LL1 muscle adopts a DA3-like morphology in a significant number of col^1 segments (Fig. 3D,E).

We then performed reciprocal experiments, i.e., examined whether expressing Col in the entire mesoderm could induce specific muscle transformations. We previously showed that Col ectopic expression in all FCs (rp298-UASCol; Dubois et al., 2007), although able to auto-activate a *col-lacZ* reporter gene, does not significantly alter the muscle pattern. To express Col earlier, including at the PC stage, we used the pan mesodermal Twist-Gal4 driver, (Greig and Akam, 1993). Vg staining of Twist > Col embryos showed that the LL1 muscle forms normally, indicating that changing the level of Col expression in the LL1 lineage does not redirect this muscle to another fate. On the contrary, the DA2 muscle adopts a DA3 or intermediate DA3/DA2 morphology in 28% (N = 35/133) and 26% (N = 37/133) of segments, respectively (Fig. 3F). The DA2 > DA3 transformation is the only specific muscle transformation that we could observe at high frequency, showing that Col ability to impose a new cell fate is strictly context-dependent, as previously observed in the central and peripheral nervous systems (Baumgardt et al., 2007; Crozatier and Vincent, 2008). The reciprocal transformations observed upon loss and gain of Col function, respectively, show that the DA2 PC is a target of reprogramming by Col and that Col activity distinguishes between the DA3 and DA2 identities (Fig. 3G).

Two steps in the selection of muscle insertion sites

The DA3 > DA2 muscle transformation in *col* mutant embryos indicated specific changes in the DA3 epidermal attachment sites. To better understand this phenotype, we first characterised the wt DA3 insertion sites, using double StripeB (SrB)/GFP staining of P9cG > GFP embryos. SrB is a specific marker for the tendon cells, which connect muscles to the epidermis (Volk and VijayRaghavan, 1994; Volohonsky et al., 2007); P9cG > GFP expression (Dubois et al., 2007) specifically labels the DA3 and, more stochastically, the DO5 muscle contours (Fig. 4A–C). Double stainings showed that, although at similar dorso/ventral (D/V) positions, the anterior insertion sites of the DA3 and DO5 muscles do not overlap. The DA3 muscle attaches to tendon cells along the segmental borders while the DO5 attaches to more internal cells. On the posterior side, the DA3 and DO5

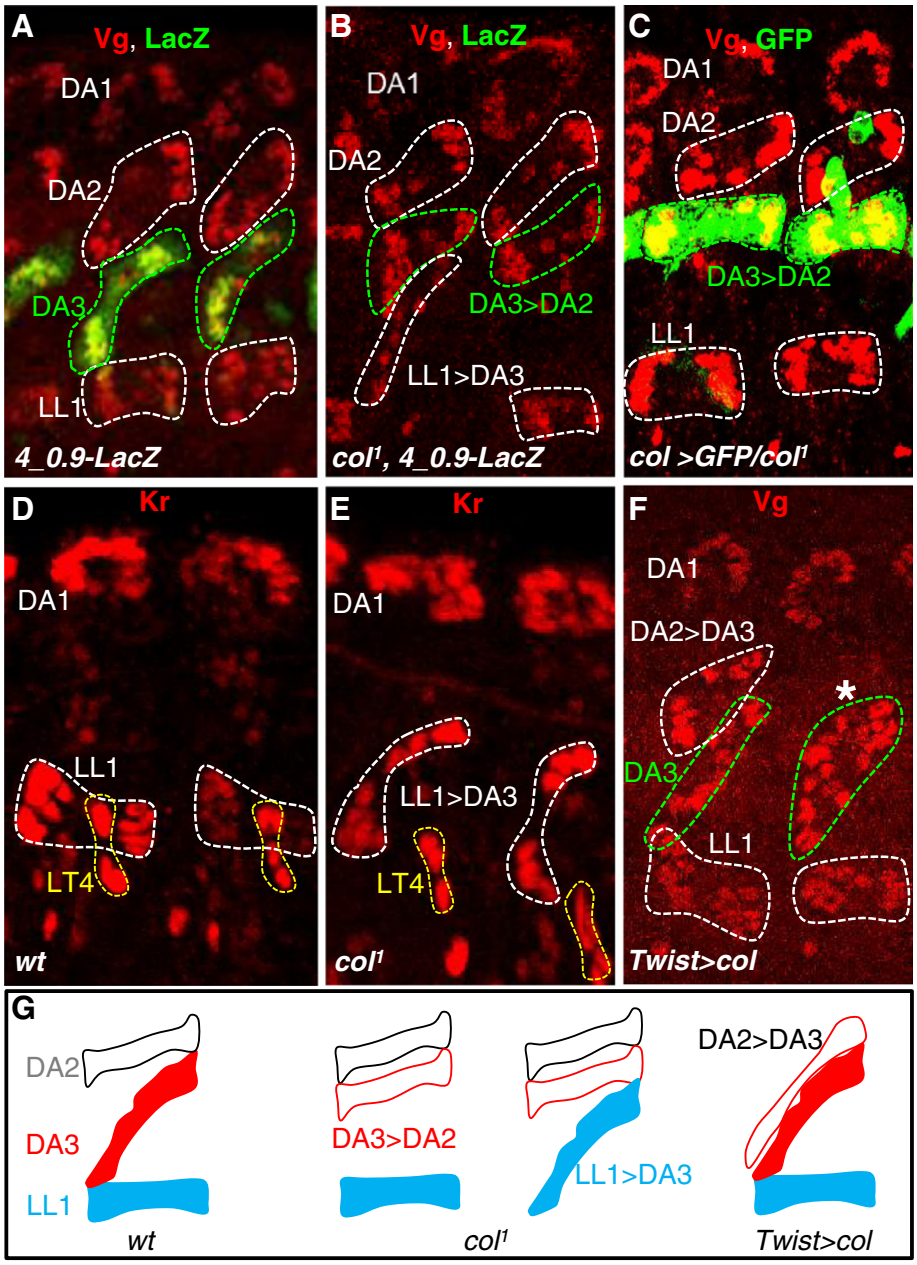


Fig. 3. Reciprocal DA3 > DA2 and DA2 > DA3 transformations in *col* loss-of-function and gain-of-function conditions. (A–F) Dorso-lateral views of stage 15 embryos stained for Vg (A–C, F) or Kr (D,E) (red), and LacZ (A–B) or GFP (C), green. Vg is expressed in the DA1, DA2 and LL1 muscles. The DA3 muscle is marked by LacZ and GFP expression, in 4_0.9LacZ and col > GFP embryos, respectively. The DA2 and LL1 muscles are circled in dotted white and the DA3 muscle in dotted green. (B,C) In *col* mutant embryos, the DA3 muscle adopts a DA2-like (DA3 > DA2) morphology. (B) One segment shows a (LL1 > DA3) transformation. (D, E) Immunostaining for Kr (red) confirms the LL1 > DA3 transformation in col^1 embryos while the LT4 muscle, circled in dotted yellow is unaffected. (F) Pan-mesodermal expression of Col (Twist > col) transforms the DA2 into a DA3-like muscle (circled green). The DA2 > DA3 transformation is either complete (asterisk, circled green) or only partial (circled white). (G) Schematic diagram of the DA3 > DA2 and LL1 > DA3 transformations in *col* loss-of-function (col^1) and reciprocal DA2 > DA3 transformation in *col* gain-of-function conditions.

attach to different tendon cells along the segmental border (Fig. 4A). Along this characterisation, we discovered that the final, acute orientation of the DA3 muscle is reached in two steps. In a first step, the DA3 muscle is attached to two distinct groups of tendon cells along the anterior segmental border and a third group along the posterior border. This three-attachment configuration gives the DA3 muscle its characteristic angled shape at stage 14 (Fig. 4B–B″). In a second step, the middle attachment site is lost, leading to a final diagonal orientation. This second step that we call the resolution step, takes place between stages 14 and 15 (Fig. 4J). In order to verify that the growing DA3 muscle forms an integrin-mediated junction with tendon cells at its intermediate attachment site, we double stained *col > GFP* embryos for GFP and the muscle specific αPS2-integrin (Bokel and Brown, 2002). This experiment indicated that αPS2-integrin accumulates at this intermediate site (stage 14; Fig. S4a), showing that a transient myotendinous junction forms. αPS2-integrin accumulation is then restricted to the DA3 final attachment sites at stage 16 (Fig. S4b), confirming that attachment site selection is a highly regulated, two-step process.

We then characterised the epidermal attachment sites of the DA3 > DA2 transformed muscle, in various combinations of *col* hypomorphic mutations, including the hypomorphic *col > GFP* allele. In heterozygous *col > GFP* embryos, a few "angled" muscles maintaining both dorsal and ventral anterior attachment sites are observed, indicating a partial DA3 > DA2 transformation (Fig. 4 C,D,G). The penetrance of this phenotype increases in homozygous *col > GFP*

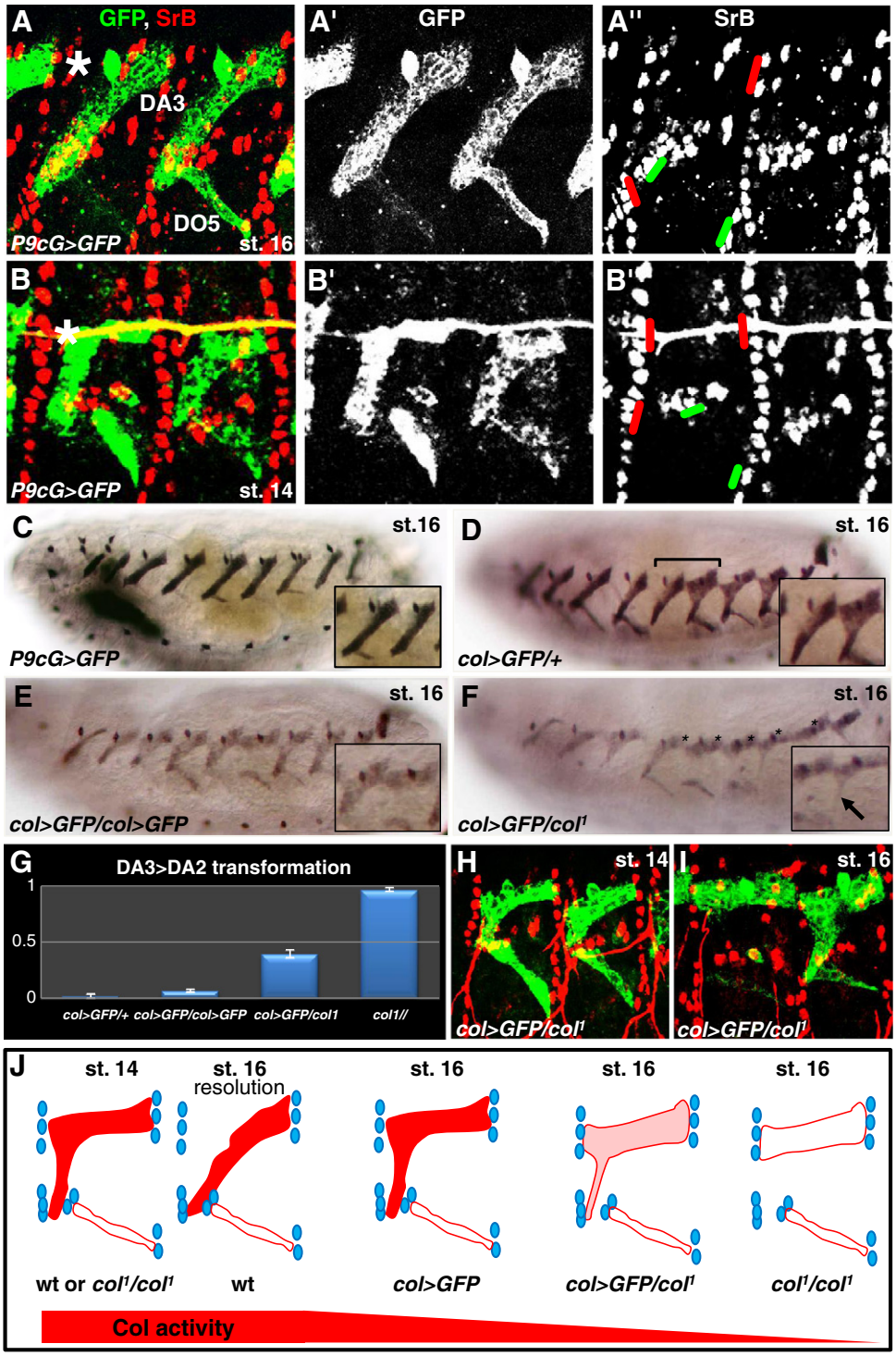
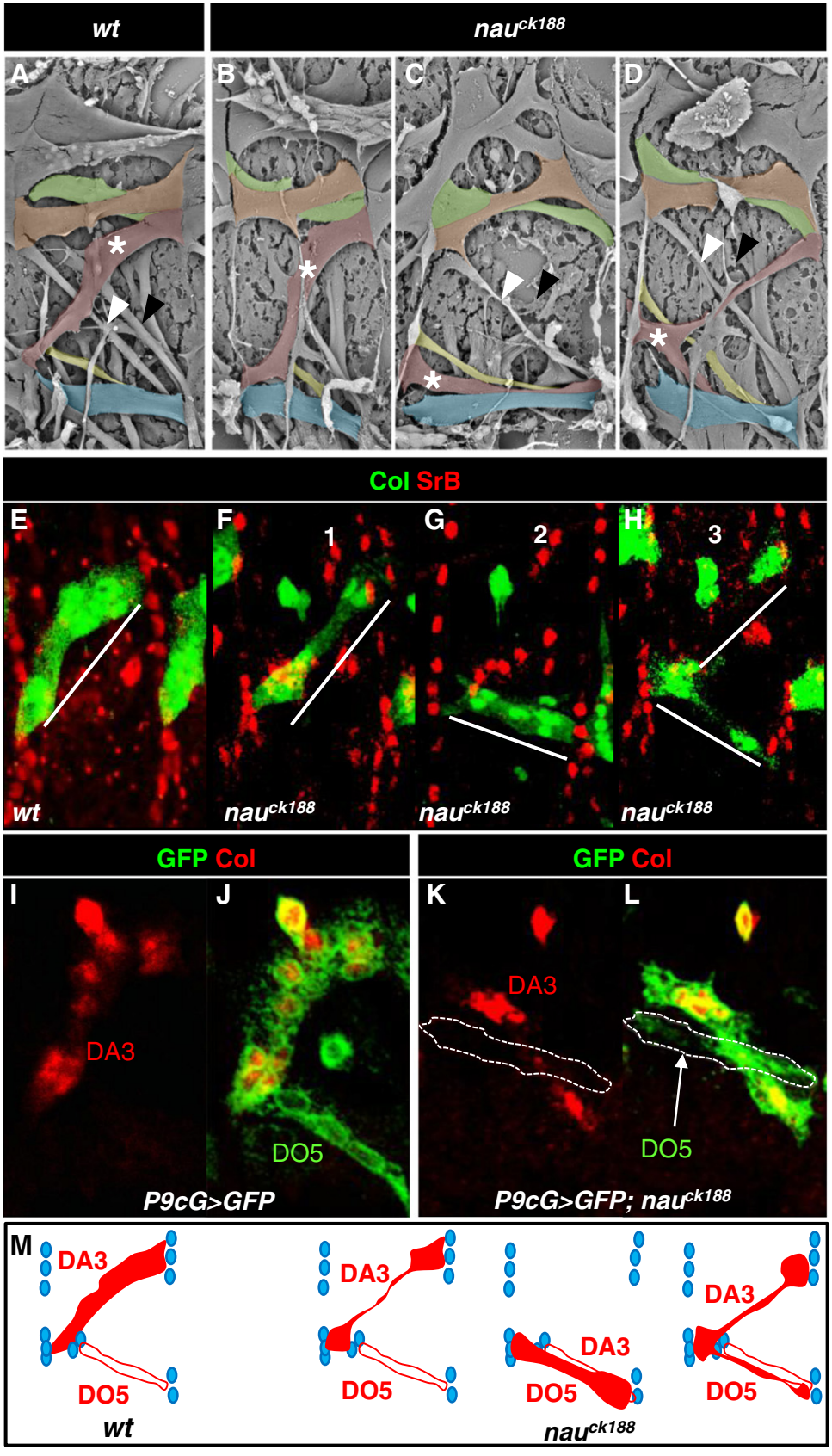


Fig. 4. Transient and final epidermal attachment sites in wt and *col* mutant embryos. (A) Stage 16, and (B), stage 14 P9cG > GFP embryos stained for GFP (DA3 and DO5 muscles; A,A′, B,B′) and SrB (A,A″, B,B″). Green and red bars in A″,B″ indicate the wt DO5 and DA3 attachment sites, respectively. The DA3 muscle shows an additional attachment site (asterisk) at stage 14. (C-F) GFP staining of the DA3 (and DO5) muscles in stage 16 P9cG > GFP embryos (C) and (D–F) various *col > GFP/col*^{1} mutant combinations, shows a progressively increasing number of DA3 > DA2 transformations; for *col*^{1} mutants, statistics are based on Vg staining, (G). Incomplete transformations maintaining three epidermal attachment sites are shown in insets in D–F. (H, I) *col > GFP/col*^{1} mutant embryos stained for SrB (red) and GFP (green). The DA3 transient attachment site observed at stage 14 (H) is maintained, leading to DA3 > DA2 re-orientation (I, left) or triangular shape muscles (I, right). (J) Diagrammatic representation of the two-steps selection of the DA3 epidermal attachment sites in wt embryos and mis-attachment phenotypes observed in different *col* mutants. The dose of *col* activity decreases from left to right (red arrow).

embryos. In addition, 7% of segments (N = 30/441) now show a complete DA3 > DA2 morphological transformation and this number increases to 40% of *col > GFP/col*^{1} segments (N = 75/189; Fig. 4E–G). In most other segments, the DA3 > DA2 muscle maintains thin projections towards the wt DA3 attachment site, suggesting again an incomplete transformation. Comparison of *col > GFP/col*^{1} embryos at stages 14 and 16 shows that the final attachment site of the DA3 > DA2 muscle corresponds to the transient DA3 attachment site that is seen in wt embryos (4B,H,I).

In summary, detailed examination of the DA3 > DA2 muscle phenotype showed that Col activity is required for attachment of the DA3 muscle to its ventral insertion site and that robustness of this

site selection requires physiological levels of Col activity. It also revealed that the final diagonal orientation of the DA3 muscle is established in two successive steps, a second, resolution step being critical (summarised in Fig. 4J). Of note, *col* mutant embryos show specific mis-attachments or triangular shapes for several muscles other than DA3 (Fig. 2 and S3), suggesting the involvement of a



resolution step for these muscles as well. Transient binding to exploratory sites during the fibre elongation process could therefore be a general feature of the developing musculature.

Nau is both required for proper muscle size and orientation of specific muscle fibres

Independent reports have described a major disorganisation of the embryonic musculature of *nau* mutant embryos (Misquitta and Paterson, 1999; Wei et al., 2007) and higher sensitivity of a subset of muscles to *nau* mutations, respectively (Balagopalan et al., 2001; Dubois et al., 2007; Keller et al., 1998). We herein re-explored the morphology of muscles in *nau* mutant embryos, using SEM analysis. It revealed a previously unnoticed phenotype which is that most fibres are much thinner in *nau* than in wt embryos (Fig. 5 and S5), confirming that, although not an essential gene, Nau plays a general myogenic regulatory function in ensuring proper fibre size. In addition, we observed many cases of either loss or mis-formation of the DA3, DO4 and DO3 muscles, while other muscles were hardly affected (Fig. 5A–D) confirming that *nau* also acts as a muscle-specific iTF (Keller et al., 1998). Puzzlingly, the severity of both phenotypes varies from embryo to embryo (Balagopalan et al., 2001; Dubois et al., 2007; Keller et al., 1998) (compare Fig. 5B–D and S5). Focusing our analysis on the DA3 muscle, we defined 4 classes of phenotypes. The DA3 muscle is either: 1) unaffected (Fig. 5A, B); 2) oriented like the DO5 muscle, (Fig. 5C); 3) attached at both the DA3 and DO5 posterior attachment sites, forming a kind of "bifid" fibre (Fig. 5D); 4) severely affected, with multiple short extensions, a phenotype that we describe as clumsy phenotype (see Fig. 6). We observed a small number of DA3>DA2 transformations (not shown), suggesting that Nau could contribute to the robustness of the handover of Col activity that is specific to the DA3/DO5 PC, consistent with Nau positively regulating *col* transcription in this lineage. To further characterise the different classes of *nau* phenotypes and calculate statistics, we used double stainings of wt and *nau* embryos for Col and Sr (Fig. 5E–H). We also counted the nuclei by staining for Mef-2 (Lilly et al., 1994; Nguyen et al., 1994) (Fig. S6). In *nau* null embryos, the DA3 muscle shows normal orientation and nuclei number (9 nuclei per DA3 muscle at stage 16; (Enriquez et al., 2010), in 51% of segments (N=115/225)). Despite having the same number of nuclei, the majority of these fibres appear much thinner than wt (Fig. 5E,F). The DA3 muscle attaches to intra-segmental tendon cells typical of a DO5 attachment and adopts the oblique orientation of the DO5 muscle in 32% (N=72/225) of segments (Fig. 5G and S6). The DA3>DO5 fibres contain only 6 nuclei on average, which is less than a wt DA3 and more than a wt DO5 (3 nuclei on average, at stage 16; Fig. S6B,E) suggesting a partial transformation. In 6% (N=14/222) of segments, the DA3 muscle displays a "bifid" DA3+DO5 morphology (Fig. 5H and S6D). In this case, the total number of nuclei is similar to wt DA3 (Fig. S6E). Finally, the DA3 muscle is absent or very poorly developed (clumsy phenotype) in 8% of segments (N=18/225), while it adopts a DA2-like orientation in 4% of segments analysed (N=9/225) (data not shown; see also Keller et al., 1998). Examination of P9cG>GFP;*nau* embryos confirmed that the DO5 muscle properly forms in the absence of Nau activity (Fig. 5I–L and S6).

In summary, our data show that, in absence of Nau activity, there is randomisation of the DA3 attachment sites between the wt DA3 and DO5 positions (Fig. 5M) as well as the formation of unstructured fibres not resembling any particular muscle, a previously observed phenotype (Keller et al., 1998). Even when correctly oriented, the *nau* mutant DA3 fibres are generally thinner than wt, despite a normal or slightly reduced number of nuclei per fibre (Fig. S6). Nau thus controls aspects of muscle fibre growth in the embryo, independently of the number of FC/FCM fusion events. *nau* phenotypes, either thinning or improper fibre orientation, vary from embryo to embryo (compare Fig. 5A–D and Fig. S5) and between segments in the same embryo (Fig. 5D), suggesting that *nau* activity may not be decisive but rather confers robustness to both generic and muscle-specific differentiation programmes.

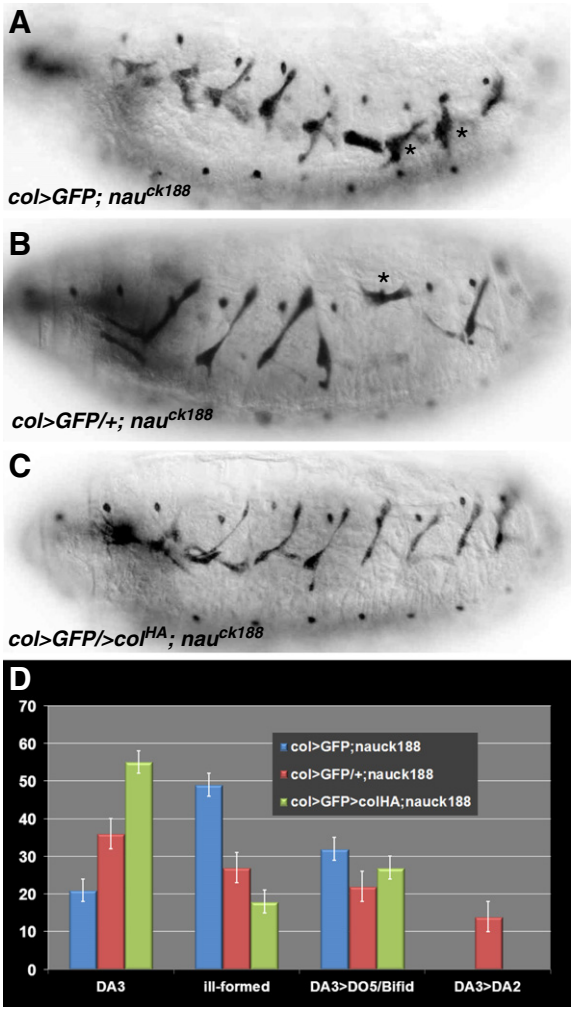


Fig. 6. Cumulative effects of *nau* and *col* mutations. (A–C) GFP staining of, (A) *col>GFP*; nau^{ck188}, (B) *col>GFP/+*;nau^{ck188} and (C), *col>GFP/>*col^{HA};nau^{ck188} stage 16 embryos. GFP expression marks the DA3 muscle. The DA3 phenotype is aggravated in double *col>GFP*;nau^{ck188} embryos, with an increasing number of ill-formed muscles (A, black asterisks). DA3>DA2 muscles are observed in *col>GFP/+*;nau^{ck188} embryos (B, black asterisk). Both phenotypes, but not the DA3>DO5 and bifid fibres phenotypes are rescued by increased levels of Col activity (C). However rescued fibres remain thinner than in col>GFP embryos (see Fig. 4 D,E). Of note, rescue is restricted to abdominal segments. (D) Histogram indicating the number of DA3, DA3>DA2, DA3>DO5+bifid, and ill-formed muscles in nau^{ck188} mutant embryos expressing increasing levels of Col. Number of segments counted: N=192 *col>GFP*; nau^{ck188}, N=129 *col>GFP/+*;nau^{ck188}; N=123 *col>GFP/>*col^{HA};nau^{ck188}.

Fig. 5. DA3 muscle phenotypes of *nau* mutant embryos. (A–D) EM-scanning views of the abdominal musculature of stage 16 wt (A) and nau^{ck188} (B–D) embryos. The internal face of one A segment is shown. Colour coding is as in Fig. 2. The white asterisk underlines the DA3 muscle. White and black arrowheads point to the DO4 and DO3 muscles, respectively, showing the frequent loss of these muscles in nau^{ck188} mutant embryos. (E–H) wt (E), and nau^{ck188} (F–H) embryos double-stained for Col and SrB. The orientation of wt and abnormal DA3 muscles is indicated by white lines. The DA3>DO5 muscle (G) contacts the same posterior tendon cells than wt DO5 (see Fig. 4). (I–L) Staining of *P9cG>GFP* (wt) and *P9cG>GFP*;nau^{ck188} embryos for Col (red) (IL) and GFP (green, J,L) showing a DA3>D05 transformation. The DO5 muscle is indicated by a white arrow in (L). (M) Schematic representation of the DA3 (red) and DO5 (circle red) muscle orientations and attachment sites in wt and nau^{ck188} mutant embryos, illustrating the reduced fibre size and stochastic orientation of the DA3 muscle in mutant embryos.

Col and Nau combinatorial control of the DA3 epidermal attachment sites

We next investigated the cumulative effect of *nau* and *col* mutations, using the hypomorphic *col>GFP* allele in order to be able to follow the morphology of the DA3 muscle. We examined trans-allelic *col>GFP/+; nau^{ck188}* and *col>GFP; nau^{ck188}* mutant combinations. Reducing the level of *col* activity in *nau* mutant embryos resulted in a strong aggravation of the DA3 muscle defects (Fig. 6A,B), with only 21% of DA3 muscles correctly oriented in *col>GFP; nau^{ck188}* compared to 51% in *nau^{ck188}* (see above) and 40% in *col>GFP/+; nauck^{188}* embryos (Fig. 6D). We also observed an increased number of clumsy muscles and DA3 muscles only attached to anterior tendon cells, phenotypes that are collectively referred to as ill-formed muscles, at the expense of DA3>DA2 muscle transformations (Fig. 6A,B,D). However, the fraction of DA3>DO5 transformations and DA3+DO5 bifid fibres, which are typical *nau* mutant phenotypes, was not significantly increased by reducing Col levels. These observations indicate a cumulative effect of *nau* and *col* mutations. Interestingly, a fraction (12%) of *col>GFP/+; nau^{ck188}* DA3 muscles adopted a DA2 morphology, the same fraction than observed in homozygous col>GFP mutant embryos (Fig. 4G), consistent with the decreased levels of *col* transcription in *nau* mutant, compared to wt embryos (Dubois et al., 2007). Nau up-regulation of *col* transcription could thus contribute to the robustness of the DA3 identity already at the progenitor stage. Restoring high levels of Col expression in *col>GFP/+; nauck^{188}* embryos, by introducing a UAS-col^{HA} transgene (*col>GFP/>col^{HA}; nauck^{188}* embryos), resulted in both full rescue of the DA3>DA2 phenotype and decreased number of ill-formed muscles, without significantly reducing the fraction of DA3>DO5 and bifid fibres (Fig. 6B–D). Together the double mutant analysis and rescue results show that combinatorial activity of *nau* and *col* is required for the correct orientation and differentiation of the growing DA3 muscle fibre (Fig. 7). Of note, even when correctly oriented, the majority of *col>GFP/>col^{HA}; nau^{ck188}* DA3 muscles remained thin (Fig. 6C), confirming that the fibre size control exerted by Nau is independent of *col* regulation.

Discussion

The larval *Drosophila* somatic musculature is made of a stereotyped set of about 30 uniquely identifiable muscles per hemisegment. Here, we show that the combinatorial activities of Col and Nau are required to establish the pattern of DL muscles and confer upon these muscles their distinctive shapes and epidermal attachment sites.

Sequential specification of the dorso-lateral muscle progenitors

Col is expressed in a promuscular cluster and the three derived PCs at the origin of DL muscles. Each of these PCs is specified at a stereotypic position and according to a precise temporal sequence, with the dorsal DA3/DO5 PC being born first and the ventral LL1/DO4 PC being born last. In addition to Col and Nau, each expresses a combination of specific iTFs, including Kr, Poxm and S59, (Fig. 1l) (Crozatier and Vincent, 1999; Dohrmann et al., 1990; Duan et al., 2007; Ruiz Gomez and Bate, 1997). Expression of a specific set of iTFs in each DL PC could thus integrate both positional and temporal cues. The textbook view is that, similar to neuroblast selection in the neuroectoderm, each muscle PC is selected via the process of lateral inhibition from an equivalence group of mesodermal cells. The parallel between neuroblast and PC selection is supported by the co-expression of the proneural gene *l(1)sc* and iTFs such as Eve or S59 in specific promuscular clusters (Carmena et al., 1995, 1998 and Baylies and Michelson,

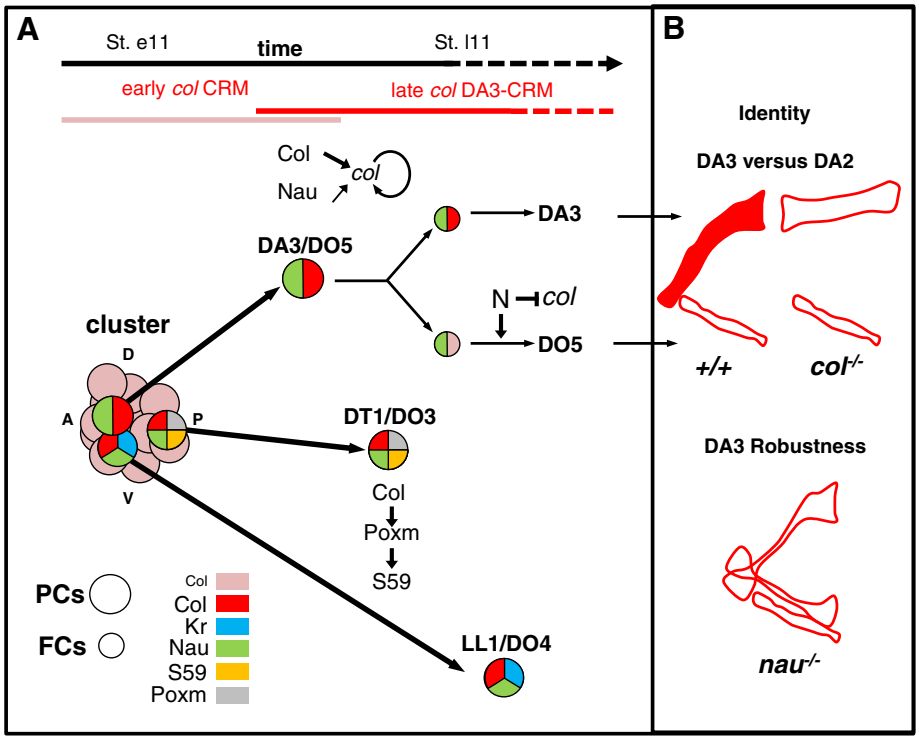


Fig. 7. The sequential and combinatorial coding of a muscle shape. (A) Schematic diagram of the DL muscle lineages. Col is expressed at low level in a promuscular cluster (pale pink) and high level in the three derived DL PCs (large colour-coded circles), which are born at fixed positions along the A–P and D–V axes. In addition to their stereotyped positioning, there exists a temporal sequence of specification of these PCs; the embryonic stages and periods of activity of the early and late *col* DA3 CRMs are indicated on a time axis (top). Each DL PC expresses different iTFs in addition to Col and Nau, such as Kr (blue), S59 (yellow) and Poxm (grey). Col activity contributes to the identity of all DL PCs, including by regulating Poxm and S59 expression in the DT1/DO3 PC and its own expression in the DA3/DO5 PC. Nau also contributes to maintain *col* expression from the PC stage. The DA3 and DO5 FCs (smaller circles) inherit both Col and Nau. B) Absence of Col activity leads to a DA3>DA2 transformation. Nau activity ensures robustness to the DA3 identity programme. This includes maintaining high level *col* transcription in this lineage (Dubois et al., 2007) and distinguishing between the DA3 and DO5 attachment sites. Thus, Col and Nau act both sequentially and combinatorially in the DA3 lineage.

2001; Frasch, 1999, for reviews). However, a deficiency of *l(1)sc* results in only minor defects of somatic muscle development (Carmena et al., 1995; Duan et al., 2007) and does not prevent the selection of the DA3/DO5 and DT1/DO3 progenitors (Crozatier and Vincent, 1999). A possibility evoked by (Duan et al., 2007), is that several PCs could be selected from large competence domains defined by expression of specific iTFs and *l(1)sc* clusters play only a limited or redundant role. Selection of the three DL PCs from a cluster of Col-expressing cells supports this view. The fact that the DA3/DO5 and LL1/DO4 PCs are born sequentially and positioned adjacent to one another (Fig. 1 and S1), suggests a reiterative selection process.

The col mutant phenotype exhibits changes in progenitor identity

The most obvious muscle pattern defects that are observed in *col* mutant embryos, are DA3>DA2 and DA3>LL1 transformations. Since the DA2, DA3 and LL1 muscles are derived from different PCs, these defects indicate changes in progenitor identity. On one side, Poxm and S59 expression in the DT1/DO3 progenitor requires Col activity. On the other side, Kr expression in the LL1/DO4 PC is independent of Col. Interestingly, Kr and S59 are expressed together in the ventral VA1/VA2 PC but, in this case, Kr regulates S59 expression (Ruiz-Gomez et al., 1997). Together, these expression data strengthen the concept of combinatorial coding of muscle identity (Bourgouin et al., 1992) at the PC stage and show that hierarchies of interactions between different iTFs are progenitor-specific.

In *Poxm* mutants, the DO3 muscle is often duplicated, likely at the expense of a DT1 muscle that is often missing (Duan et al., 2007). In *S59* mutant embryos, the DO3 and DT1 sibling muscles share ventral attachment sites and form a single syncitium in a fraction of segments (Knirr et al., 1999). Since Col acts upstream of Poxm and S59 in the DT1/DO3 progenitor, we expected the *col* mutant phenotype to overlap with the *poxm* and *S59* phenotypes. It may not be so simple, however, since the DT1 muscle is absent only in a small fraction of segments in *col* mutant embryos. Interestingly, while the DA3 muscle is transformed into a DA2-like muscle in absence of Col activity, the LL1 muscle can adopt a DA3 morphology The LL1 muscle is misoriented in *col* as well as in *Kr* mutant embryos (Dohrmann et al., 1990; Ruiz-Gomez et al., 1997). Together, these muscle reorientation phenotypes suggest that there is a range of possible attachment sites for each elongating DL muscle and that the final pattern results from a global combinatorial control. The propensity of elongating muscles to explore several attachment sites (see below), could explain why a coordinate, global regulation by combinations of iTFs is essential. The term regulatory state has been used to describe the total set of active transcription factors in a given cell at a given time (Peter and Davidson, 2011). In essence, each PC iTF code is an example of a regulatory state. The loss of one iTF reveals an alternative regulatory state and PC identity, suggesting that a given iTF is able to exert its activity only in the presence of other specific iTFs. A global analysis of this mutual dependency now requires the identification of all DL iTFs, including those expressed in the DO3, DO4 or DO5 muscles.

The myogenic functions of Nau, the Drosophila MyoD ortholog

Nau differs from other well characterised iTFs, in that it is expressed in most, if not all FCs (Wei et al., 2007), before being restricted to specific muscle precursors (Dubois et al., 2007; Keller et al., 1998; Michelson et al., 1990). SEM analysis shows that most muscles are much thinner in *nau* mutant than wt embryos. Detailed examination of the mutant DA3 muscle showed that, despite being thinner, it contained a number of nuclei close to normal. *nau* activity is thus required for embryonic muscle fibre size, but not the muscle fusion programme, per se. Whether Nau directly or indirectly regulates the synthesis and/or assembly of myofibril proteins remains to be determined. As first noted by Keller et al. (1998), DL muscles, including the DA3 muscle, are more severely affected. Taken together, these data lead us to conclude that Nau performs both general myogenic functions and specific functions in selected muscle lineages. A different threshold level of MRF activity might be needed to initiate myogenesis in different trunk and craniofacial muscles (reviewed by (Sambasivan et al., 2011)). The different Nau functions in establishing the *Drosophila* muscle pattern suggest that Nau activity is, in part conditioned by interactions with other iTFs such as Col.

Temporal, combinatorial coding and robustness of muscle shape

Co-expression of Nau and Col in the DA3/DO5 progenitor provides a good model to challenge the concept of combinatorial control of muscle identity (Dubois et al., 2007). While transformed towards a DA2 muscle in absence of Col activity, the DA3 muscle adopts the morphology of its sibling, DO5 muscle in absence of Nau. Thus, while co-expressed in the DA3/DO5 PC, Col and Nau act at different steps in the DA3 lineage. We propose the following regulatory cascade (Fig. 7): Col expression in a large cluster of myoblasts and the three derived PCs, under control of an early CRM and Hox activity (Enriquez et al., 2010), defines a domain of competence for DL muscle development. Col activity, either upstream, and/or in parallel to other iTFs, contributes to confer each DL progenitor its particular identity. The restricted ability of Col in maintaining its own expression in the DA3 FC, by direct binding to a late, DA3-specific CRM (Dubois et al., 2007), reveals a context-dependence provided by the iTF combination specific to the DA3/DO5 PC. This PC-specific handover process may explain why the DA3 muscle is the most frequently affected in *col* mutant embryos. Asymmetric division of each DL PC generates two FCs with different regulatory states (Carmena et al., 1998; Ruiz Gomez and Bate, 1997). Whereas two DA3 and two DO5 muscles form in Notch (N) loss- and gain of function conditions, respectively (Crozatier et al., 1996), Nau confers robustness to the DA3 versus DO5 differentiation programme. This Nau function involves positive regulation of *col* transcription in the DA3 syncytium nuclei (Dubois et al., 2007) and is independent of Nau function in ensuring normal fibre size.

In conclusion, our data show that the sequence of expression and combinatorial activities of Col and Nau are required to establish the pattern of DL muscles and confer upon the DA3 muscle its distinctive size and epidermal attachment sites. Identification of the gene targets of this combination is now essential to link a sequence of regulatory states to the architecture of a specific *Drosophila* muscle. Interestingly, a recent report suggests that EBF cooperates with MyoD in driving aspects of differentiation in *Xenopus* muscle cells, suggesting that there may be an ancient, evolutionarily conserved, transcriptional relationship between the COE/EBF and MyoD gene families (Green and Vetter, 2011).

Muscle targeting of specific tendon cells

Embryonic muscles connect to the chitinous exoskeleton of the developing embryo via tendon cells, which are specialised epidermal cells (Becker et al., 1997; Schnorrer and Dickson, 2004). Proper attachment of muscles requires the specific targeting of tendon cells at segmental or intra-segmental, stereotypic positions. The general view is that growing myotubes extend filopodia at their two ends, in search of attachment sites, and that muscle extension ceases when muscles have reached their targeted tendon cells. Some muscle guidance components have been described, such as the Derailed receptor tyrosine kinase for the lateral transverse muscles and the Robo and Robo2 receptors, the transmembrane protein Kon-Tiki and its associated intracellular signalling protein dGrip for ventral-longitudinal muscles (Callahan et al., 1996; Kidd et al., 1999; Schnorrer et al., 2007; Swan et al., 2004). How the precise matching

of specific muscles to specific tendon cells is achieved, however, is far from being understood (Schnorrer and Dickson, 2004; Schnorrer et al., 2007; Schweitzer et al., 2010). SEM analysis and phalloidin staining of *col* mutant embryos showed many mis-oriented muscles, suggesting targeting defects. Many fibres showed more than two attachment sites to the epidermis, however, a phenotype difficult to reconcile with a bipolar extension of muscle precursors until they connect to the epidermis. Rather, the observation that the wt DA3 muscle is transiently attached to three sites, before acquiring its fully extended bipolar morphology, indicates the existence of an exploratory step, followed by a resolution step that selects the final attachments sites. The allelic series of *col* phenotypes, which revealed many triangular shape fibres, indicates a defect in the resolution process, without ruling out that ventral elongation of the DA3 myofibre is also defective. Terminal differentiation of tendon cells is dependent upon their interaction with muscles (Schweitzer et al., 2010; Yarnitzky et al., 1997) and tendon cells could play a role in the resolution step. Triangular shape LO1 muscles were previously observed in mutants for *dgit*, which encodes a GTPase activator protein that is involved in myotube guidance (Bahri et al., 2009). Based on the *dgit* phenotype, and our own observations, we propose that the migratory path of muscles towards their targeted tendon cells can involve exploratory attachment to tendon cells along this path. Deciphering how the final, stereotyped, pattern is controlled now requires the identification of how various iTF combinations differentially regulate guidance cues.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.12.018.

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